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The Effects of Calcium Ions on the Activities of Trehalase, Hexokinase, Phosphofructokinase, Fructose Diphosphatase and Pyruvate Kinase from Various Muscles

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1. The effects of Ca^{2+} on the activities and regulatory properties of trehalase, hexokinase, phosphofructokinase, fructose diphosphatase and pyruvate kinase from vertebrate red and white muscle and insect fibrillar and non-fibrillar muscle have been investigated. These muscles were selected because of the possible difference in the role of glycolysis in energy production in the vertebrate muscles, and the possible difference in the role of Ca^{2+} in the control of contraction in the two types of insect muscle. An increase in Ca^{2+} concentration from 0.001 μM to 10 μM did not modify the activities nor did it modify the regulatory properties of these enzymes from these various muscles. 2. Concentrations of Ca^{2+} above 0.1 mM inhibited the activities of hexokinase and phosphofructokinase from the different muscles. It has been suggested that this inhibition may provide the basis for a theory of regulation of glycolysis (Margreth *et al.*, 1967). If phosphofructokinase is located within the sarcoplasmic reticulum, its activity will be inhibited when the muscle is at rest, but the release of Ca^{2+} from the reticulum during contraction will lead to a stimulation of its activity and hence an increase in glycolytic flux. The distribution of hexokinase and phosphofructokinase in the various cell fractions of these muscles was very variable. In particular, both enzymes were present almost exclusively in the 100000*g* supernatant fraction in the extracts of insect flight muscles. Thus there is no correlation between the properties of the enzymes and their distribution in muscle. 3. It is concluded that Ca^{2+} does not control the activities of the important regulatory enzymes of glycolysis in muscle. It is suggested that in some muscles the sensitivity of the control mechanism at the level of phosphofructokinase to changes in the concentration of AMP may be increased by a process known as 'substrate-cycling'.

Stimulation of mechanical activity in muscle increases the rate of glycolysis, which can be explained as follows. The increase in ATP utilization due to contraction causes a small decrease in the steady-state concentration of ATP and this, via the reaction catalysed by adenylate kinase, produces a much larger increase in the concentration of AMP. The latter stimulates the activity of phosphofructokinase (EC 2.7.1.11), an important regulatory enzyme in glycolysis, so that the flux through the pathway is increased (for review see Newsholme, 1970). Evidence in support of this theory has been obtained from studies with the isolated perfused rat heart. Anaerobiosis or heart work increases the flux through the phosphofructokinase reaction by ninefold and fourfold, when the changes in the contents of AMP are approximately fourfold and twofold respectively (see Regen *et al.*, 1964; Opie *et al.*, 1971). These changes in AMP are quantitatively consistent with control of phosphofructokinase by a simple reversible binding mechanism (see Newsholme, 1972; Newsholme & Start, 1972). However, some experimental findings appear to be inconsistent with this theory. Electrical stimulation of the isolated frog sartorius muscle or the cat hind-limb preparation *in situ* results in contraction and increased glycolytic flux, but there are no statistically significant changes in the concentrations of the adenine nucleotides (Karpatkin *et al.*, 1964; Helmreich *et al.*, 1965; Wilson *et al.*, 1967). Furthermore, the rate of glycolysis in the flight muscles of the blowfly is increased about 100-fold when the insect takes to flight, but the increase in the content of AMP is only 2.5-fold (Sacktor & Hurlbut, 1966). The magnitude of the change in AMP is not sufficient to account for the increase in phosphofructokinase activity in the flight muscles of the blowfly, if phosphofructokinase is regulated by AMP via a simple reversible binding mechanism.

The link between electrical stimulation and the contraction of a muscle is the rapid change in the intracellular distribution of Ca^{2+}. Stimulation promotes a release of Ca^{2+} from the sarcoplasmic

reticulum and the concentration of Ca^{2+} in the sarcoplasm increases from about 0.01 μM to about 1.0 μM. This is sufficient to activate the myofibrillar ATPase and initiate contraction. Relaxation is brought about by the uptake of Ca^{2+} by the sarcoplasmic reticulum so that the concentration in the sarcoplasm is decreased to about 0.01 μM (for reviews see Hasselbach, 1964; Weber, 1966; Ebashi & Endo, 1968). It has been suggested that this change in the sarcoplasmic Ca^{2+} concentration may control not only contraction but also the process of glycolysis (see Helmreich *et al.*, 1965). This hypothesis predicts that the activities of the enzymes that may regulate glycolysis (i.e. phosphorylase, EC 2.4.1.1; hexokinase, EC 2.7.1.1; phosphofructokinase, fructose diphosphatase, EC 3.1.3.11; pyruvate kinase, EC 2.7.1.40) should be increased by Ca^{2+}. There is substantial evidence that the activity of phosphorylase *b* kinase, which controls the activity of phosphorylase in muscle, is increased by Ca^{2+} in the range 0.01–1.0 μM (Ozawa *et al.*, 1967; Brostrom *et al.*, 1971), and therefore electrical stimulation of muscle increases the rate of glycogenolysis. Thus the hexose monophosphate concentration is raised and it can be proposed that this would lead to activation of phosphofructokinase. However, this simple control mechanism, linking electrical stimulation and the process of glycolysis, is not satisfactory. Glycogen stores in muscle are limited so that, for sustained mechanical activity, blood glucose must be the primary substrate for glycolysis. Consequently, hexokinase (EC 2.7.1.1) and phosphofructokinase must be controlled independently of phosphorylase. Furthermore, adrenaline activates phosphorylase and increases the hexose monophosphate concentration to a greater extent than does electrical stimulation in frog sartorius muscle, but it activates glycolysis to a lesser extent (Helmreich *et al.*, 1965); in other words the activation of phosphofructokinase due to electrical stimulation is independent of the change in concentration of fructose 6-phosphate. To test the hypothesis that Ca^{2+} regulates glycolysis in muscle the effects of Ca^{2+} on the activities of hexokinase, phosphofructokinase, pyruvate kinase and fructose diphosphatase have been investigated. The studies have been carried out with the enzymes isolated from vertebrate red and white muscles, because the role of glycolysis in the provision of energy is different in these muscles (see Crabtree & Newsholme, 1972). Furthermore, the enzymes isolated from fibrillar and non-fibrillar insect flight muscle have been studied, because the role of Ca^{2+} in the control of contraction in these two muscles is somewhat different (Pringle, 1967). Since insect muscles were to be included in this study, the effect of Ca^{2+} on trehalase activity was investigated, because this is another regulatory enzyme in the glycolytic pathway in these muscles (see Sacktor & Wormser-Shavit, 1966).

Materials and Methods

Chemicals, enzymes and animals

All chemicals, enzymes and animals were obtained from sources given previously (Crabtree & Newsholme, 1972; Newsholme *et al.*, 1972). Succinate was purchased from the Boehringer Corp. (London) Ltd. (London W5 2TZ, U.K.).

Preparation of homogenates

Vertebrate muscles were rinsed in ice-cold extraction medium, dried on filter paper, weighed rapidly and cut into small pieces. They were homogenized first in a Silverson homogenizer and then in a power-driven Teflon–glass homogenizer. Insect muscles were carefully dissected from the thorax, chilled in a glass beaker on ice, weighed and homogenized in a ground-glass homogenizer. Homogenization was carried out with 10–50vol. of extraction medium, except for phosphofructokinase from insect muscle, which was homogenized in no more than 10vol. of extraction medium (see Crabtree & Newsholme, 1972). For the assays of trehalase, hexokinase, fructose diphosphatase and pyruvate kinase the extraction medium consisted of 50mM-Pipes,* 10mM-$MgCl_2$, 10mM-mercaptoethanol, 0.5mM-EDTA and 0.5mM-EGTA, at a final pH of 7.8. For the assay of phosphofructokinase the extraction medium consisted of 50mM-Tris, 10mM-$MgCl_2$, 10mM-mercaptoethanol, 0.5mM-EDTA and 0.5mM-EGTA, at a final pH of 8.2. For the assay of the activities of fructose diphosphatase and hexokinase, the extracts were dialysed against the extraction medium for 2h at 0°C to lower the concentrations of inhibitory compounds present in the extract (e.g. AMP, ADP, glucose 6-phosphate). The activities of phosphofructokinase and pyruvate kinase are high so that the dilution of the extract in the cuvette lowers sufficiently the concentration of ATP.

Enzyme assays

Distilled water was passed through an Elgastat portable deionizer (type C-403) to remove traces of metal ions. The concentration of Ca^{2+} in the assay buffers has been maintained at about 0.001 μM or about 10 μM by the use of the Ca^{2+}–EGTA buffer system (Portzehl *et al.*, 1964): two stock solutions were prepared, 100mM-EGTA and 100mM-EGTA plus 100mM-$CaCl_2$, each adjusted to pH7.1 with KOH. A final concentration of 2mM-EGTA was used in all the assay buffers. Hexokinase was assayed radiochemically (Newsholme *et al.*, 1967): the

* Abbreviations: Pipes, piperazine-*NN'*-bis (2-ethanesulphonic acid); EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid.

incubation medium consisted of 20mM-Pipes, 5mM-mercaptoethanol, 2mM-$MgCl_2$, 0.2mM-[^{14}C]glucose (6μCi/ml), 1mM-ATP and various concentrations of glucose 6-phosphate and Ca^{2+}–EGTA at pH7.1. Other enzymes were assayed spectrophotometrically at 340nm with a Gilford recording spectrophotometer, model 240. Trehalase was assayed by coupling the product formation (glucose) to the reduction of NADP with hexokinase and glucose 6-phosphate dehydrogenase. The incubation medium consisted of 20mM-Pipes, 5mM-mercaptoethanol, 6mM-$MgCl_2$, 2.5mM-ATP, 0.2mM-NADP and various concentrations of Ca^{2+}–EGTA at pH7.1; glucose 6-phosphate dehydrogenase and hexokinase (10μg of each) were added to 2ml of incubation medium in the cuvette. Phosphofructokinase was assayed in a buffer system consisting of 20mM-Pipes, 200mM-KCl, 5mM-$MgCl_2$, 0.5mM-KCN, 1mM-fructose 6-phosphate, 0.1mM-NADH and various concentrations of ATP and Ca^{2+}–EGTA at pH7.1; aldolase (50μg), glycerol phosphate dehydrogenase (5μg), triose phosphate isomerase (5μg) and a sample (1–5μl) of extract (see Crabtree & Newsholme, 1972) were added to 2ml of incubation medium in the cuvette. (The coupling enzymes were dialysed for 2h before use.) Fructose diphosphatase was assayed in the following incubation medium: 20mM-Pipes, 5mM-mercaptoethanol, 6mM-$MgCl_2$, 0.1mM-fructose diphosphate and various concentrations of AMP and Ca^{2+}–EGTA at pH7.1; glucose 6-phosphate dehydrogenase (10μg) and glucose phosphate isomerase (10μg) were added to 2ml of incubation medium in the cuvette. Pyruvate kinase was assayed in the following incubation medium: 160mM-triethanolamine, 5mM-ADP, 0.05mM-phosphoenolpyruvate, 25mM-Mg^{2+}, 67mM-KCl, 0.14mM-NADH and various concentrations of ATP and Ca^{2+}–EGTA at pH7.2; lactate dehydrogenase (25μg) was added to 2ml of incubation medium in the cuvette. Succinate dehydrogenase was assayed by the method described for proline dehydrogenase (Crabtree & Newsholme, 1970) except phenazine methosulphate was not present and 5mM-succinate replaced the proline.

All the enzymes were assayed at 25°C and the activities are expressed as μmol of product formed/min per g fresh wt. of muscle.

Preparation of cell fractions

The procedure was based on that used in the preparation of sarcoplasmic reticulum by Hasselbach & Makinose (1963) and that used for the preparation of mitochondria (see Chappell & Perry, 1954; Van den Berg, 1967). The extraction medium consisted of 20mM-Tris–HCl, 100mM-KCl, 5mM-$MgCl_2$, 0.5mM-EDTA, 0.5mM-EGTA, 1mM-ATP, 1mM-ADP and 1mM-AMP at pH7.9. Vertebrate muscle was homogenized in this medium for 5s in a Silverson homogenizer. Insect muscle was homogenized manually in a Teflon–glass homogenizer. Homogenates were centrifuged at 500*g* for 10min at 0°C to remove myofibrils and cell debris. The supernatant was passed through muslin gauze and then centrifuged at 8000*g* (insect-muscle homogenate) or 15000*g* (vertebrate-muscle homogenate) for 10min at 0°C. The pellet was resuspended in the above medium and assumed to represent the mitochondrial fraction. The supernatant was centrifuged at 100000*g* for 1h at 0°C. The pellet was resuspended in the above medium and assumed to represent the reticulum fraction. The 100000*g* supernatant is termed 'supernatant' in Table 7. Before enzyme assay, all fractions were sonicated for two 10s periods in a MSE ultrasonic disintegrator (100W) at 20kHz and the solutions were maintained as near as possible to 0°C.

Results

The effects of Ca^{2+} on the enzymes have been investigated by using crude extracts of muscle without any purification or other treatment (apart from dialysis in some cases). Any purification procedure was considered undesirable since it might cause modification of the properties of the enzyme under study. For the assay of hexokinase and phosphofructokinase, ATP-regenerating systems were not included in the assay buffers, so that both ADP and AMP would be produced from ATP by enzymes present in the crude extracts of the muscles. This was considered desirable since any effect of Ca^{2+} may require the presence of ADP (or AMP). Thus hexokinase activities were measured in the presence of ADP, a known inhibitor of the enzyme, and this may explain why the activities reported in this paper are low in comparison with other published results (see Crabtree & Newsholme, 1972). Furthermore, most of the enzyme activities were assayed at pH7.1 (or 7.2), because this may be reasonably close to the physiological pH. Since phosphofructokinase and trehalase have pH optima approximately 8.2 and 6.0 respectively this may explain why the activities observed in this work were low.

Glucose 6-phosphate inhibition of hexokinase from various muscles

Before investigation of the effects of Ca^{2+} on the glucose 6-phosphate inhibition of hexokinases in extracts of insect flight muscles and pigeon pectoral muscle, it was necessary to establish that the enzymes from these muscles were inhibited by this compound. Table 1 shows that all the hexokinases were inhibited by low concentrations of glucose 6-phosphate. The enzymes from the flight muscles of the blowfly, cockroach and locust and from the pigeon pectoral muscle were very sensitive to glucose 6-phosphate,

Table 1. *Effect of glucose 6-phosphate on the activities of hexokinase from various muscles*

The concentration of Ca^{2+} was approx. 0.001 μM. Percentage inhibitions by glucose 6-phosphate are given in parentheses.

Animal	Muscle	Concn. of added glucose 6-phosphate (mM)	Hexokinase activity (μmol/min per g fresh wt. of muscle)				
		...	0.0	0.03	0.06	0.15	0.30
Cockroach (*Periplaneta americana*)	Flight (non-fibrillar)		1.84	1.15 (38)	0.85 (53)	0.40 (78)	0.11 (94)
Locust (*Schistocerca gregaria*)	Flight (non-fibrillar)		3.57	1.89 (44)	1.20 (66)	0.57 (84)	0.09 (97)
Fleshfly (*Sarcophaga barbata*)	Flight (fibrillar)		5.2	3.5 (33)	2.2 (57)	1.0 (80)	0.4 (91)
Bumble-bee (*Bombus hortorum*)	Flight (fibrillar)		24.4	21.4 (12)	19.3 (21)	12.7 (48)	6.0 (75)
Laboratory rat	Heart		0.78	0.67 (13)	0.63 (19)	0.36 (53)	0.25 (68)
Pigeon (*Columbia liva*)	Pectoral		0.13	0.10 (23)	0.085 (35)	0.051 (61)	0.021 (84)

whereas those from the bumble-bee flight muscle and the rat heart were less sensitive.

Effects of 0.001 μM- and 10 μM-Ca^{2+}

In resting muscle the concentration of Ca^{2+} in the sarcoplasm is about 0.01 μM and this is increased to about 1.0 μM to initiate contraction. Therefore the Ca^{2+} concentrations tested in this investigation were approximately 0.001 μM (the lowest concentration produced by the EGTA buffer system) and 10 μM (representing the highest concentration that might be obtained in the sarcoplasm in the muscle fibre). There was no difference in the activities of trehalase from the flight muscles of the fleshfly and bumble-bee at these two extreme concentrations of Ca^{2+}. Similarly, there was no difference in the activities of hexokinase, phosphofructokinase, fructose diphosphatase and pyruvate kinase from a variety of insect flight muscles and vertebrate muscles (the results for hexokinase and phosphofructokinase are shown in Tables 2 and 3). Furthermore this change in Ca^{2+} concentration did not modify the glucose 6-phosphate inhibition of hexokinase from these muscles, nor did it influence the glucose 6-phosphate inhibition in the presence of 8 mM-P_i (Table 2). Similarly there was no modification of the inhibitory effects of ATP on phosphofructokinase (Table 3) or on pyruvate kinase from various muscles, and the inhibitory effects of AMP on fructose diphosphatase from various muscles was not modified.

Effects of Ca^{2+} concentrations above 0.1 mM

Ca^{2+} concentrations above 0.1 mM inhibited the activities of hexokinase, phosphofructokinase and fructose diphosphatase from various insect flight and vertebrate muscles (Tables 4, 5 and 6).

Activities of succinate dehydrogenase, hexokinase and phosphofructokinase in cell fractions

The distribution of succinate dehydrogenase was primarily mitochondrial in all the muscles investigated (Table 7). This enzyme provides some indication of the contamination of the reticulum and supernatant fractions with mitochondria. The distribution of hexokinase and phosphofructokinase varied considerably with the different muscles. In frog muscle about 50% of both hexokinase and phosphofructokinase was associated with the supernatant fraction and the remainder of the phosphofructokinase activity was associated with the reticulum (Table 7; see also Margreth *et al.*, 1967). A similar distribution for phosphofructokinase was observed for the pigeon pectoral muscle, but almost all the hexokinase activity was present in the supernatant fraction. In rat heart most of the phosphofructokinase was associated with the supernatant fraction, whereas hexokinase

Table 2. *Effect of* 0.001 μM- *and* 10 μM-Ca^{2+} *on the activities of hexokinase from various muscles*

Animal	Muscle	Concn. of added glucose 6-phosphate (mM)	Concn. of added P_i (mM)	Hexokinase activity (μmol/min per g fresh wt. of muscle) 0.001 μM-Ca^{2+}	10 μM-Ca^{2+}
Cockroach	Flight	0.0	0	2.5	2.3
(*Periplaneta americana*)	(non-fibrillar)	0.05	0	1.2	1.4
Locust	Flight	0.0	0	3.0	3.0
(*Schistocerca gregaria*)	(non-fibrillar)	0.1	0	1.06	1.04
		0.1	8	1.14	1.18
Fleshfly	Flight (fibrillar)	0.0	0	4.28	4.23
(*Sarcophaga barbata*)		0.05	0	2.73	2.60
		0.05	8	2.70	2.40
Bumble-bee	Flight (fibrillar)	0.0	0	24.1	22.5
(*Bombus hortorum*)		0.05	0	15.6	15.6
		0.05	10	15.6	14.7
Laboratory rat	Heart	0.0	0	1.17	1.13
		0.1	0	0.61	0.65
		0.1	8	0.66	0.61
Pigeon	Pectoral	0.0	0	0.18	0.15
(*Columbia liva*)		0.1	0	0.15	0.10

Table 3. *Effects of* 0.001 μM- *and* 10 μM-Ca^{2+} *on the activities of phosphofructokinase from various muscles*

Animal	Muscle	Concn. of added ATP (mM)	Phosphofructokinase activity (μmol/min per g fresh wt. of muscle) 0.001 μM-Ca^{2+}	10 μM-Ca^{2+}
Locust	Flight (non-fibrillar)	0.1	7.5	7.8
(*Schistocerca gregaria*)		1.0	7.8	7.8
		5.0	0.9	0.6
		10.0	0.9	0.9
Fleshfly	Flight (fibrillar)	0.1	16.0	12.8
(*Sarcophaga peregrina*)		1.0	24.0	22.5
		5.0	15.2	19.0
		10.0	10.4	12.8
Bumble-bee	Flight (fibrillar)	0.1	12.5	11.2
(*Bombus agrorum*)		1.0	22.5	22.0
		5.0	9.2	10.8
		10.0	6.4	6.8
Dogfish	White	0.1	0.3	0.3
(*Scylliorhinus canicula*)		1.0	10.2	9.6
		5.0	12.8	13.4
		10.0	10.9	10.9
Frog	Thigh	0.1	5.5	5.6
(*Rana temporaria*)		1.0	8.7	7.5
		5.0	5.2	6.5
		10.0	1.0	1.0
Laboratory rat	Heart	0.1	3.1	—
		1.0	5.2	—
		5.0	1.9	2.9
		10.0	1.3	1.8

Table 4. *Effects of concentrations of Ca^{2+} above 0.1 mM on activities of hexokinase from various muscles*

Animal	Muscle	Concn. of added glucose 6-phosphate (mM)	Concn. of Ca^{2+} (mM) ...	Hexokinase activity (μmol/min per g fresh wt. of muscle)					
				0.01	0.71	1.0	2.0	5.0	10.0
Cockroach (*Periplaneta americana*)	Flight (non-fibrillar)	0.0		2.25	—	1.95	1.52	1.15	1.07
		0.03		1.45	—	1.30	1.30	1.02	0.55
Locust (*Schistocerca gregaria*)	Flight (non-fibrillar)	0.0		3.57	—	2.84	2.80	2.34	1.54
		0.06		1.20	—	1.00	0.91	0.84	0.55
Fleshfly (*Sarcophaga barbata*)	Flight (fibrillar)	0.0		5.6	—	4.4	3.9	3.3	2.4
		0.15		1.0	—	0.8	0.5	0.3	0.2
Bumble-bee (*Bombus hortorum*)	Flight (fibrillar)	0.0		24.4	—	24.4	22.7	18.5	12.7
		0.06		19.3	—	14.9	14.7	11.8	6.6
Laboratory rat	Heart	0.0		0.74	0.61	0.28	0.20	0.15	—
		0.15		0.34	0.29	0.17	0.13	0.08	—
Pigeon (*Columbia liva*)	Pectoral	0.0		0.18	0.12	0.08	0.05	0.05	—
		0.1		0.15	0.10	0.07	0.05	0.03	—

Table 5. *Effects of concentrations of Ca^{2+} above 0.1 mM on the activities of phosphofructokinase from various muscles*

Animal	Muscle	Concn. of added ATP (mM)	Concn. of Ca^{2+} (mM) ...	Phosphofructokinase activity (μmol/min per g fresh wt. of muscle)						
				0.01	0.1	0.5	1.0	2.0	5.0	10.0
Locust (*Schistocerca gregaria*)	Flight (non-fibrillar)	0.2		6.75	—	—	—	2.5	—	1.4
		2.5		3.5	—	1.7	—	1.5	—	0.8
Frog (*Rana temporaria*)	Thigh	0.2		15.4	12.2	11.5	10.7	10.6	5.4	4.2
Laboratory rat	Heart	0.2		3.6	2.8	2.6	2.2	2.1	1.2	0.7

Table 6. *Effects of concentrations of Ca^{2+} above 0.1 mM on the activities of fructose diphosphatase from various muscles*

Animal	Muscle	Concn. of Ca^{2+} (mM) ...	Fructose diphosphatase activity (μmol/min per g fresh wt. of muscle) 0.01	0.3	0.6	1.2
Cockroach (*Periplaneta americana*)	Flight (non-fibrillar)		0.63	0.42	0.23	0.10
Locust (*Schistocerca gregaria*)	Flight (non-fibrillar)		0.43	0.24	0.07	0.02
Bumble-bee (*Bombus hortorum*)	Flight (fibrillar)		7.2	2.0	1.3	1.3
Waterbug (*Lethocerus cordofanus*)	Flight (fibrillar)		0.83	0.47	0.28	0.13
Frog (*Rana temporaria*)	Thigh		0.22	0.13	0.08	0.03
Domestic fowl (*Gallus gallus*)	Pectoral		1.5	0.8	0.6	0.3

Table 7. *Activities of hexokinase, phosphofructokinase and succinate dehydrogenase in cell fractions from various muscles*

Details of the preparation of cell fractions are given in the Materials and Methods section.

Animal	Muscle	Cell fraction	Enzyme activities (μmol/min per g fresh wt. of muscle) Hexokinase	Phospho-fructokinase	Succinate dehydrogenase
Locust (*Schistocerca gregaria*)	Flight (non-fibrillar)	Crude extract	13.10	9.0	2.00
		Mitochondria	0.45	0.01	1.40
		Reticulum	0.32	0.10	0.08
		Supernatant	12.50	10.3	0.01
Fleshfly (*Sarcophaga barbata*)	Flight (fibrillar)	Crude extract	14.9	5.8	14.0
		Mitochondria	1.9	<0.01	5.5
		Reticulum	2.8	<0.01	3.3
		Supernatant	21.1	5.9	<0.01
Frog (*Rana temporaria*)	Thigh	Crude extract	1.90	12.80	—
		Mitochondria	0.20	0.16	—
		Reticulum	0.25	5.60	—
		Supernatant	0.65	7.50	—
Pigeon (*Columbia liva*)	Pectoral	Crude extract	0.58	13.60	1.15
		Mitochondria	0.02	0.64	0.53
		Reticulum	0.02	5.90	0.18
		Supernatant	0.53	8.10	0.01
Laboratory rat	Heart	Crude extract	1.50	6.40	4.3
		Mitochondria	0.65	0.13	4.5
		Reticulum	0.38	0.51	1.0
		Supernatant	0.84	7.9	<0.01

was distributed between all three fractions. However, for the two insect flight muscles investigated, both enzymes were detected almost exclusively in the supernatant fraction (Table 7).

Discussion

If Ca^{2+} is to provide a common regulatory mechanism for simultaneous control of both contraction and the pathway that generates energy for the contraction

(i.e. glycolysis), the activities of the regulatory enzymes of the pathway might be modified directly by Ca^{2+} in the concentration range 0.001–10 μM. The results of the present investigation show that the activities of trehalase, hexokinase, phosphofructokinase, fructose diphosphatase and pyruvate kinase are not modified by Ca^{2+} in this concentration range. However, these findings alone are not sufficient to justify the conclusion that the regulatory enzymes of glycolysis are not controlled by Ca^{2+}. First, it is important to ensure that the effects of Ca^{2+} are tested on these enzymes at substrate concentrations that are not saturating. (An effect of Ca^{2+} on the K_m of the enzyme for its substrate may not be observed at saturating concentrations.) Secondly, it is important to test the effect of Ca^{2+} on the regulatory properties of these enzymes, since Ca^{2+} could change the enzyme activity by modification of such properties. The concentrations of substrates in the incubation buffers were usually less than saturating for all the enzymes, except fructose diphosphatase. [The latter enzyme has a very low K_m for its substrate and is likely to be saturated with substrate in the muscle; see Opie & Newsholme (1967).] The properties of the enzymes that are relevant to metabolic control are as follows: hexokinase is inhibited by glucose 6-phosphate and this inhibition is relieved by P_i (see England & Randle, 1967); phosphofructokinase is inhibited by ATP and this inhibition is relieved by AMP (Passonneau & Lowry, 1962; Mansour, 1963); fructose diphosphatase is inhibited by AMP (Opie & Newsholme, 1967); pyruvate kinase is inhibited by ATP (Tanaka *et al.*, 1967). The effectiveness of these compounds in causing inhibition of the respective enzymes was not modified by changing the Ca^{2+} concentration from 0.001 μM to 10 μM.

In relation to the role of glycolysis for energy production or the role of Ca^{2+} in the contractile mechanism, muscle can be divided into at least four classes: red and white vertebrate muscle, and insect fibrillar and non-fibrillar muscle. It was considered important to investigate the effects of Ca^{2+} on glycolytic enzymes from these four classes of muscles for the following reasons. In vertebrate white muscle the anaerobic conversion of glycogen into lactate provides most of the energy for short bursts of mechanical activity; in red muscle glucose is converted into pyruvate, most of which is oxidized by the tricarboxylic acid cycle (and the electron-transport chain) and this provides the energy for contraction. It might be argued that since white muscle is almost totally dependent upon anaerobic glycolysis for energy production the control of glycolysis by Ca^{2+} should be more important in white than in red muscle. Insect fibrillar muscle is less dependent upon Ca^{2+} for the control of the contraction–relaxation cycle than non-fibrillar muscle (see Pringle, 1967), so that Ca^{2+} might be less important in the control of glycolysis in fibrillar muscle. However, 0.001–10 μM-Ca^{2+} did not modify the activities or the regulatory properties of the glycolytic enzymes isolated from any of these four classes of muscle.

Margreth *et al.* (1967) have observed that phosphofructokinase from frog leg muscle is inhibited by 1 mM-Ca^{2+}, and that a large proportion of this enzyme could be sedimented with the sarcoplasmic reticulum fraction. They suggested that most of the phosphofructokinase is localized within the reticulum in this muscle so that, under resting conditions, the activity would be inhibited by the high local concentration of Ca^{2+} (10–20 mM). Electrical stimulation of the muscle would lower the concentration of Ca^{2+} within the reticulum and lead to an activation of phosphofructokinase. This would increase glycolysis and provide energy for contraction. Since hexokinase is inhibited by 1 mM-Ca^{2+} the control mechanism could apply to this enzyme. Such a hypothesis, however, is difficult to test directly because of the problems associated with the precise intracellular localization of enzymes (e.g. the phosphofructokinase that is associated with the reticulum might be inside or outside the vesicles). An indirect approach was therefore used to test this hypothesis. Since concentrations of Ca^{2+} above 0.1 mM inhibited the activities of hexokinase and phosphofructokinase from the four different types of muscle described above (Tables 4 and 5), the hypothesis predicts that these enzymes would be associated with the sarcoplasmic reticulum in all the muscles. An investigation into the distribution of these enzymes in fibrillar and non-fibrillar insect flight muscles and vertebrate red and white muscles showed that there was no consistent pattern. Only in pigeon pectoral and frog thigh muscle was any large proportion of the activity of phosphofructokinase associated with the reticulum fraction. Indeed, in rat heart and in the insect flight muscles most of the phosphofructokinase and hexokinase activities were associated with the supernatant fraction.

The results of the present investigations do not provide any support for the hypothesis that physiological changes in the concentrations of Ca^{2+} in the sarcoplasm can modify the activities of the regulatory enzymes of glycolysis. Consequently, to explain the marked stimulation of phosphofructokinase activity in vertebrate skeletal muscle and in some insect flight muscles, either the enzyme is regulated by some unknown factors or the sensitivity of the regulatory mechanism to changes in AMP concentration must be increased. It is suggested that the existence of a 'substrate-cycle' between fructose 6-phosphate and fructose diphosphate, which is catalysed by the simultaneous activities of phosphofructokinase and fructose diphosphatase, is re-

sponsible for an increase in the sensitivity of the rate of fructose 6-phosphate phosphorylation to changes in the concentration of AMP in these muscles (see Newsholme & Crabtree, 1970, 1973). In this case very small changes in AMP concentration, which may not be detected experimentally, could cause large modifications in the rate of fructose 6-phosphate phosphorylation in muscle.

It must be borne in mind that changes in Ca^{2+} concentration could modify the rate of glycolysis by regulating the rate of glucose transport into the muscle. Unfortunately this possibility cannot yet be tested, since the process of transport cannot be studied *in vitro*. Nonetheless, stimulation of transport by Ca^{2+} could not explain the marked increase in flux through the phosphofructokinase reaction that is accompanied by a small change in the concentration of fructose 6-phosphate under some conditions of mechanical activity (e.g. insect flight; see Sacktor & Wormser-Shavit, 1966).

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